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IMPROVING ASCOCHYTA RABIEI RESISTANCE IN CHICKPEA

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PERENNIAL CHICKPEA: POTENTIAL FOR IMPROVING *ASCOCHYTA RABIEI* RESISTANCE

Final Report on:

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M. Lulsdorf[1], T. Warkentin[1], B. Tar'an[1], A. Diederichsen[2], S. Banniza[1]

[1]Crop Development Centre, University of Saskatchewan, 51 Campus Drive, Saskatoon SK S7N 5A8
[2]Agriculture & Agri-Food Canada, Plant Gene Resources of Canada, Science Place, Saskatoon

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A. ABSTRACT

Ascochyta blight caused by the fungus *Ascochyta rabiei* is one of the major challenges for sustainable chickpea production in Saskatchewan. The project objective was to find superior sources of blight resistance in wild perennial *Cicer* species and to transfer this resistance into the cultivated chickpea via hybridization. In a two year field trial, all wild perennial accessions showed significantly higher blight resistance under heavy disease pressure and no fungicide application. Accession *C. anatolicum* PI 383626 was identified as having the best resistance rating of 1.8 as compared to the most resistant cultivated variety CDC Frontier (6.1). Crossing wild accessions with the cultivated chickpea resulted in hybrid embryo development. Yet, hybrid plants could not be regenerated from these crosses indicating that the rescue procedure needs further improvements.

B. EXECUTIVE SUMMARY

Ascochyta blight, caused by the fungus *Ascochyta rabiei*, is one of the major constraints to chickpea production in western Canada due to reductions in yield and seed quality as well as increased production costs. A high level of resistance has not been found by any researchers in the world in current cultivars and germplasm collections. Furthermore, recent research at the Crop Development Centre showed that there has been a shift in the *A. rabiei* population in Saskatchewan to more aggressive strains, indicating that the partial resistance available in current cultivars could break down over time, and that the frequency of disease incidence and severity could increase in the future. Hence, better sources of blight resistance are urgently needed.

The objective of this project was to find sources with superior resistance to ascochyta blight in wild perennial *Cicer* species and to transfer this resistance into the cultigen by crossing wild accessions with chickpea cultivars (interspecific hybridization).

In a two year field trial, all perennial accessions tested were shown to have ascochyta blight resistance superior to our partially resistant control cultivar, CDC Frontier, under high disease pressure and no fungicide application. The accession *C. anatolicum* PI 383626 had an average ascochyta resistance rating of 1.8 (using the Horsefall-Barratt scale from 0 = immunity to 11 = 100% diseased), compared to 6.1 for CDC Frontier.

Experiments aimed at incorporating this resistance into the cultivated species via interspecific hybridization continued and further improvements to the procedure were made. Crossing focused on developing hybrids with our most resistant species *C. anatolicum* and *C. oxyodon*, on testing the possibility of using *C. macracanthum* and *C. microphyllum* for bridge crossing, as well as on improving the rescue medium needed for the continued development of globular embryos. Over 3000 crosses were made during the summer of 2009 but hybrid plants could not be recovered.

A contributing project was funded by the Saskatchewan Pulse Growers in the spring of 2009 on "Chickpea regeneration: development of rescue media using hormone profiling analysis of anthers and young ovules". Due to a labour shortage (two summer students that were hired quit just prior to May 1st) and infrastructure problems (no growth chambers were available due to chiller problems in the phytotron) the analysis of young ovules has not yet been carried out. Dr. Sue Abrams from the Plant Biotechnology Institute has made a suitable growth chamber available to this project at PBI starting in December of 2009 and a student has been hired to carry out the research as planned. Results should be available in the spring of 2010. Since hormones are especially critical during early stages of embryo development, the information garnered from this experiment will help in the development of a more suitable rescue medium for wide crosses.

A new project on 'Infection studies of *Ascochyta rabiei* on wild chickpea germplasm' funded by the Saskatchewan Pulse Growers is currently underway. This study will determine whether the resistance mechanism in perennial species is very durable and thus more desirable (i.e. of a non-host-type), based on a gene-for-gene interaction (i.e. less durable and prone to resistance breakdown) as in our current cultivars, or has a completely different mechanism. If the outcome of the infection study shows that the resistance found in perennial chickpea is very durable, and the hormone analysis of young ovules leads to an improved rescue protocol, then efforts should continue to transfer blight resistance from wild perennials to the cultivated species.

C. ABBREVIATIONS

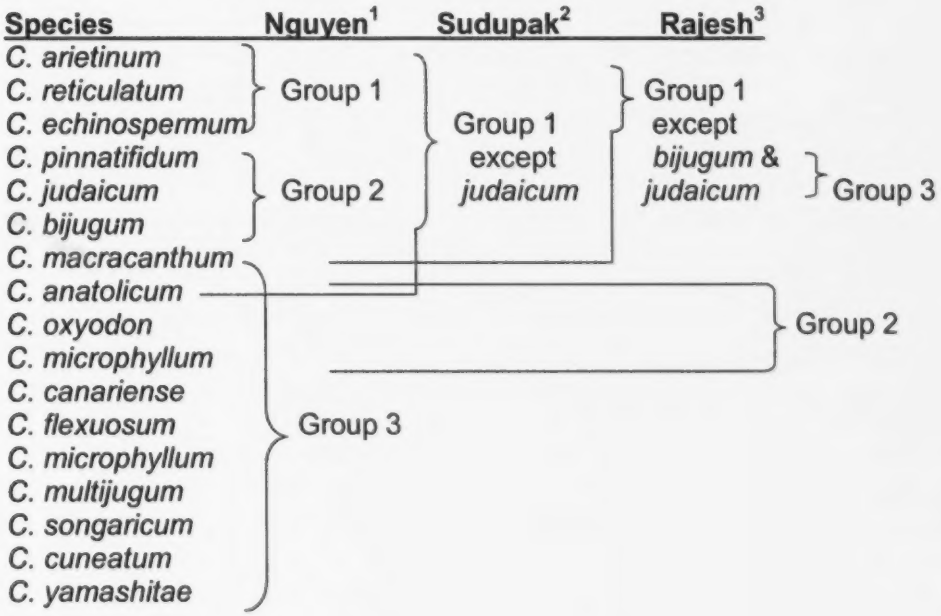
AAFC	Agriculture & Agri-Food Canada, Saskatoon
CDC	Crop Development Centre, University of Saskatchewan, Saskatoon
C-DNA	DNA content of the complete chromosome complement with chromosome number n
CIAT	Centro Internacional de Agricultura Tropical, Columbia
CIGAR	Consultive Group on International Agriculture Research
ICRISAT	International Crops Research Institute for the Semi-Arid Tropics, Patancheru, India
PGRC	Plant Gene Resources of Canada, Agriculture & Agrifood-Canada, Saskatoon
USDA	United States Department of Agriculture, Pullman, WA, USA
DCICA	Dichloroisocyanuric acid
GA_3	Gibberellic acid
Glu	Glutamine
IAA	Indole acetic acid
NAA	Naphthalene acetic acid
TNT	calcium hypochlorite

D. TECHNICAL REPORT

1. Project Background

Ascochyta blight is one of the major constraints to chickpea production in western Canada. This disease contributed to a drastic reduction in acreage in Saskatchewan between 2002 - 2004 due to reduced yields, seed quality and increased production costs (Pearse 2001). No immunity was found in current cultivars and germplasm collections (Warkentin *et al* 2003, Pande *et al* 2005). Furthermore, recent research showed that there has been a shift in the *A. rabiei* population in Saskatchewan to more aggressive strains (Vail and Banniza 2008), suggesting that the frequency of disease incidence and severity could increase in the future. Hence, better sources of blight resistance, such as those from wild perennial species are urgently needed.

There are 34 perennial *Cicer* species with a diploid chromosome number of 2n = 16 (van der Maesen 1987). Information about the cross-ability of perennial with cultivated *Cicer* species, as well as their level of ascochyta blight resistance is sparse. Regarding *Ascochyta rabiei*, Muehlbauer *et al* (1994) reported that accessions from *C. anatolicum* and *C. songaricum* have shown long term resistance in their disease nursery. Singh *et al* (1991) found one accession of *C. montbretii* highly resistant in their disease survey.
Fig. 1. Simplified genetic relationships in the genus *Cicer*



[1]Nguyen *et al* 2004
[2]Sudupak *et al* 2002
[3]Rajesh *et al* 2003

Several researchers have attempted to determine the genetic relationship between the cultivated chickpea (*Cicer arietinum* L) and its wild relatives. A simplified overview of

these relationships is presented in Fig 1. Polymorphism studies by Rajesh *et al* (2003) grouped *C. acanthophyllum*, *C. macracanthum*, *C. pungens* and *C. nuristanicum* in the same cluster as *C. arietinum*. Sudupak *et al* (2002) determined that *C. montbretii*, *C. isauricum*, *C. anatolicum* and *C. incisium* were clustered with the annual species *C. pinnatifidum*, *C. judaicum* and *C. bijugum* based on RAPD marker analysis. Nguyen *et al* (2004) reported that the perennial *C. oxyodon* was the most closely related to the annual species. Ohri (1999) noticed that the *C. songaricum* karyotype had close similarity to the *C. arietinum* and *C. reticulatum* karyotype. However, only three attempts of crossing perennial *Cicer* species with the cultigen are published in the literature. Mallikarjuna (2001) reported obtaining globular embryos in crosses of *C. arietinum* with C. *canariense*. Mercy *et al* (1975) found pre-fertilization barriers in crosses with *C. songaricum* and Babb & Muehlbauer (2005) determined that embryos aborted during early growth phases in crosses with *C. anatolicum*.

Terminology

Fig 2 shows an opened chickpea flower consisting of anthers containing pollen, and the pistil consisting of stigma, style, and ovary. The stigma is the sticky end of the style which collects the pollen.

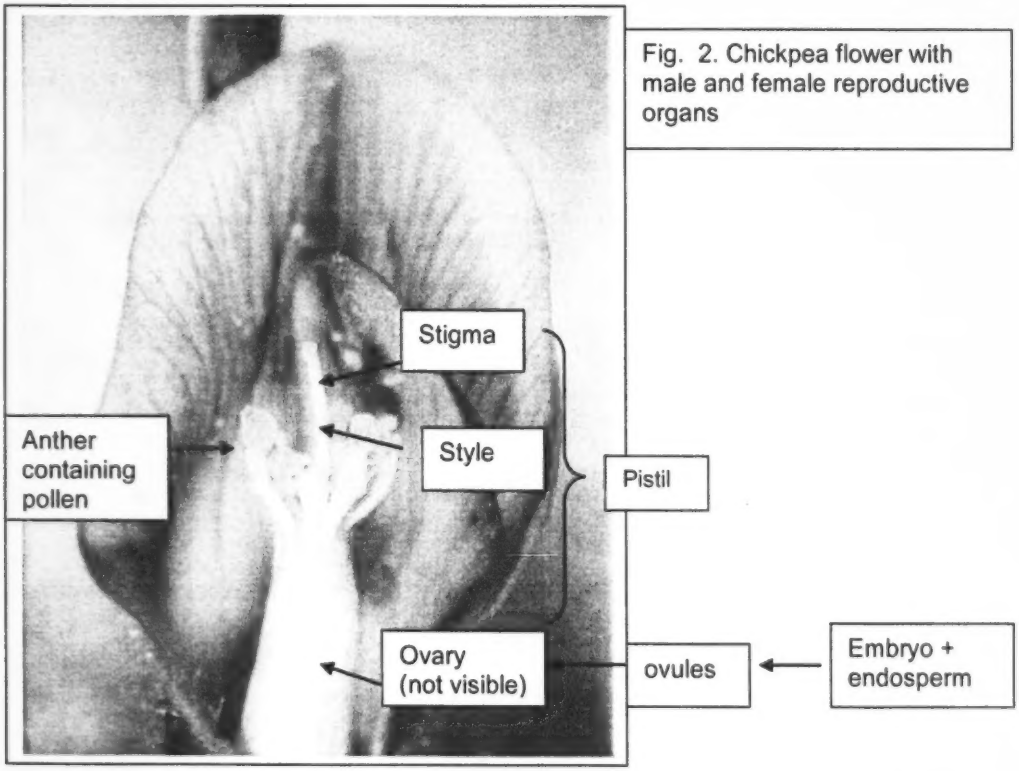


Fig. 2. Chickpea flower with male and female reproductive organs

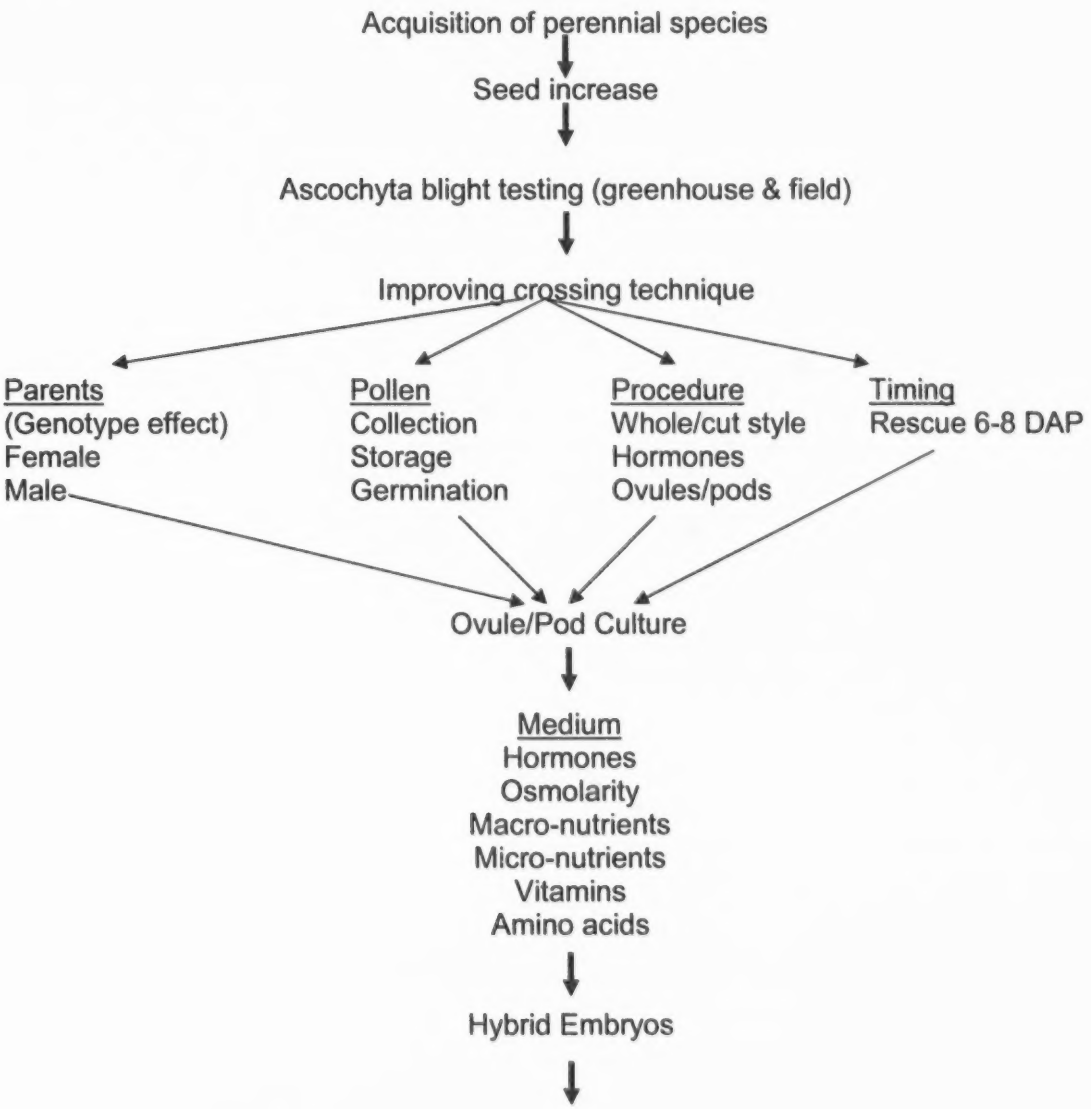
Attached to the bottom part of the style is the ovary (not visible). Ovaries contain egg cells which need to be fertilized by the pollen. Hence, pollen germinates on the stigma,

grows through the style until it reaches the ovary and fertilizes the egg cells. The ovary contains ovules which develop into seeds once fertilized. Each ovule contains an embryo and its endosperm (nutritive tissue).

2. Project Objectives

The objective of this project was to identify sources with superior resistance to ascochyta blight in wild perennial *Cicer* species and to transfer this resistance into the cultigen by crossing the wild species with cultivated chickpea varieties (interspecific hybridization). An overview of the different aspects of this project is presented in Fig. 3.

Fig. 3. Flow chart overview of the perennial project



Regeneration
Shoot development
Root Development
Adaptation to soil
Fertility/Sterility

↓

Testing and Backcrossing

The final project year focused on the second repeat of the ascochyta blight field trial, the development of a more efficient rescue medium for hybrid embryo development and on optimizing the timing of rescue depending on the different crosses i.e. 5 - 6 days for crosses involving *C. anatolicum* and *C. oxyodon* and 8 -10 days for crosses with *C. macracanthum* and *C. microphyllum*.

3. Results and Discussion

3.1. Identifying *Cicer* species

Two major obstacles were encountered in the use of wild perennial *Cicer* species in the chickpea breeding program. On the one hand, the availability of *Cicer* germplasm was very limited. The USDA station in Pullman (Washington, USA) was the only place worldwide that successfully managed a perennial *Cicer* nursery and offered seeds for distribution. However, even the USDA had only 9 out of 34 perennial species available. On the other hand, misclassifications of species in world collections are unfortunately not uncommon. We encountered this problem in our collection with several species but especially with the two *C. songaricum* accessions (PI 99053 and 599074) that were both identified as belonging to *C. microphyllum* with only one plant being the exception and actually being *C. songaricum*. Since this species was supposedly highly resistant to *Ascochyta* blight it was unfortunate that it could not be tested in our field trial because seed collection from this plant did not start until last year.

The problem of correctly identifying species was addressed in two ways based on botanical and complementary DNA (C-DNA) since neither method is completely accurate. The botanical identification of each plant in our nursery was carried out by Dr. van der Maesen, an expert *Cicer* taxonomist, as well as by sending herbarium samples to Dr. I. Seferova the N.I. Vavilov Research Institute in St. Petersburg, Russia. The result of this investigation was presented in the 2008 report and a conference paper titled 'Challenges in preserving, communicating and utilizing the diversity of wild perennial species of the genus *Cicer* L' was presented at the Sinskaya conference in St. Petersburg in November 2009.
A second method involving cytogenetic characterization in terms of relative nuclear DNA content (C-DNA) may help in this respect, as a novel predictive tool. This work was carried out in collaboration with Dr. Sergio Ochatt from INRA, France. The C-DNA

content has been determined for the cultivated and some of the annual chickpea (see Royal Botanical Gardens Kew website). The cultivated chickpea is a diploid (2n) with an approximate genome size of 750 Mbp as assessed by Feulgen method. The C-value describes the nuclear DNA content based on the chromosome number (n) for each species and can be quickly measured via flow cytometry (Ochatt 2008,Greilhuber *et al* 2005). Accessions tested are listed in Table 1.

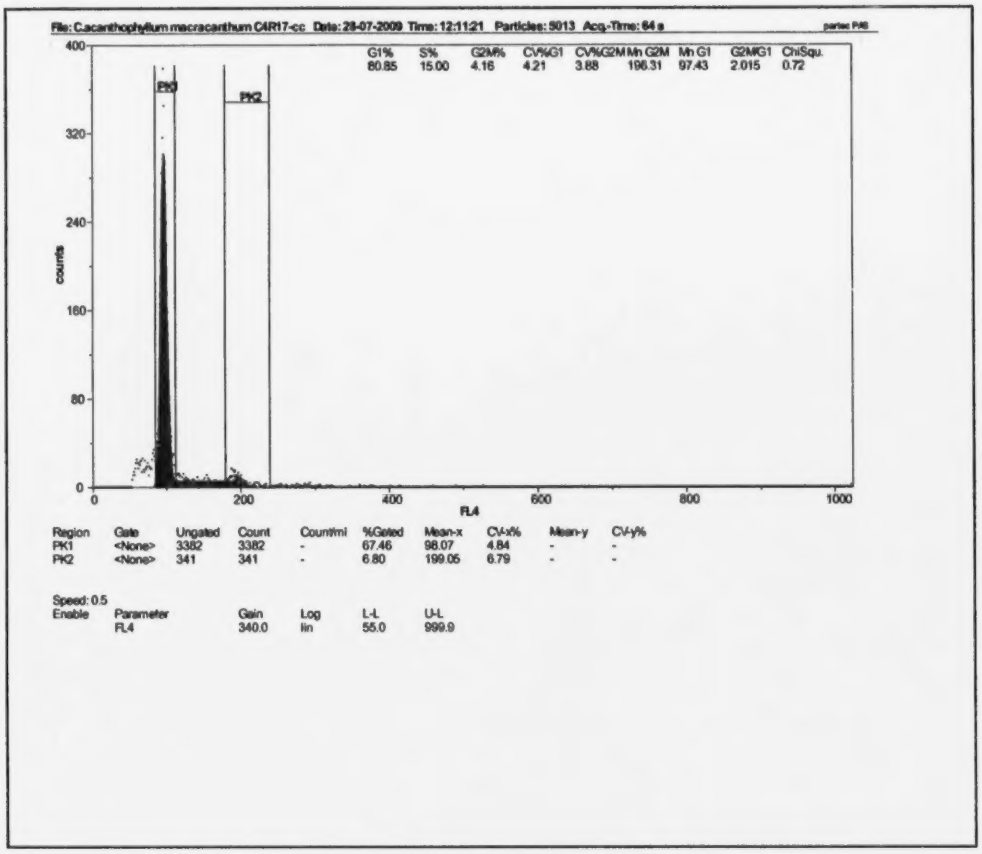
Table 1. *Cicer* species analyzed via flow cytometry

PI	Species ID	Received as	G1*
	C. arietinum	CDC Xena	102.38
	C. arietinum	ICCV2	96.75
PI599087	anatolicum	anatolicum	98.99
PI383626	anatolicum	anatolicum	89.90
PI14524	flexuosum	flexuosum	105.18
PI14524	songaricum	flexuosum	104.59
PI599074	songaricum	songaricum	101.00
PI599053	microphyllum	songaricum	99.52
PI599074	microphyllum	songaricum	84.02
PI599082	microphyllum-H	microphyllum	82.05
PI599093	microphyllum	microphyllum	80.78
PI599082	microphyllum	microphyllum	51.15
PI593719	microphyllum	microphyllum	93.18
PIW611516	micro/song	multijugum	81.38
PIW611516	unknown	multijugum	93.27
PI599084	macracanthum	microphyllum	72.07
PI599081	macra/micro	macracanthum	59.22
PI599081	macracanthum	macracanthum	89.97**
PI599079	macracanthum	macracanthum	87.84
PI599079	acanthophyllum	macracanthum	97.52
PI604497	nuristanicum	nuristanicum	82.60
PI561084	oxyodon-diff	oxyodon	80.62
PI561103	oxyodon	oxyodon	72.79
PI557453	canariense	canariense	114.28

*Cell cycle progression is typically divided into phases G1, S, G2 (also called interphase) and M (mitosis). DNA replication occurs exclusively during S phase, such that G2-phase cells have twice the cellular DNA content compared to the G1 cells. After completion of mitosis the cell divides generating two daughter cells (G1) each having half the DNA content of the G2 cell.

**Example for use of C-DNA values for distinguishing species. Accessions highlighted in red show the difference between true '*macracanthum*' species with C-DNA values of 89.97 and 87.84 whereas the '*macracanthum*' species with a C-DNA value of 97.52 was identified as belonging to '*acanthophyllum*' on the basis of botanical criteria.

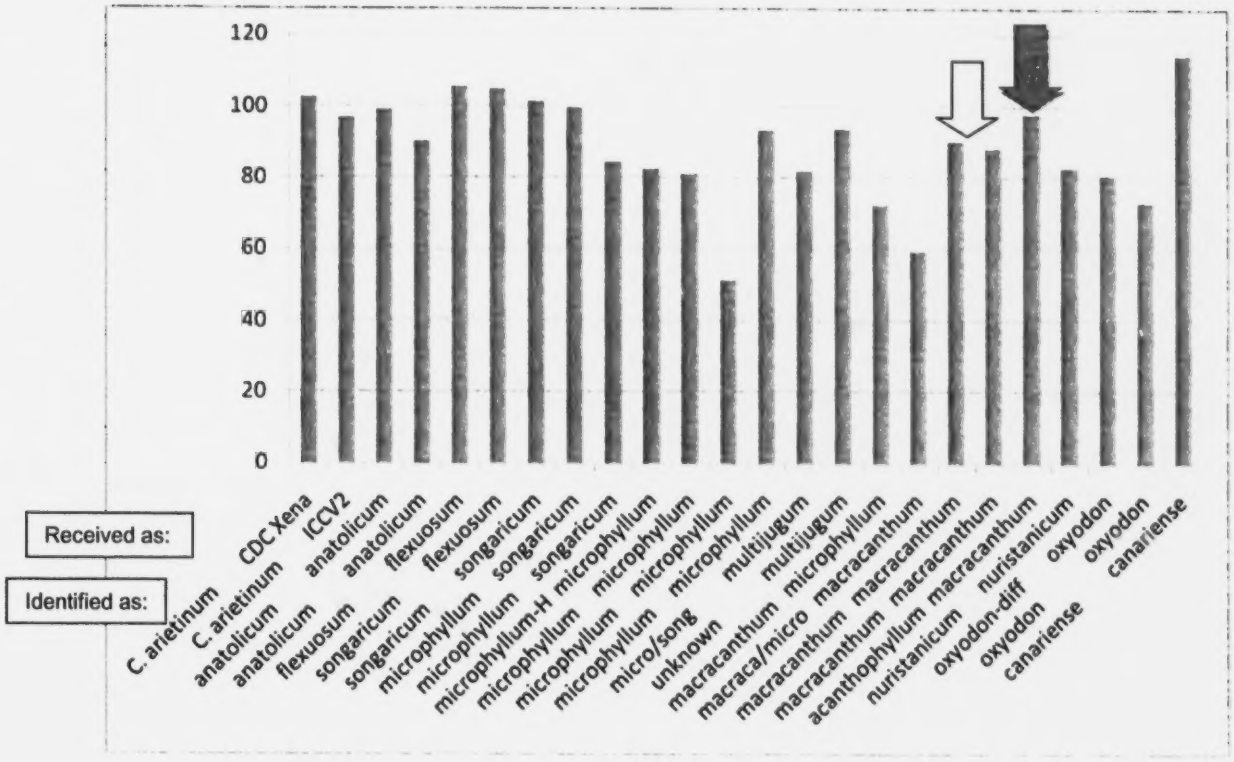
Fig. 4. Example of flow cytometry profile showing *C. macracanthum* (C4R17) plant that was identified as *C. acanthophyllum* on the basis of botanical characteristics.


File: C.acanthophyllum macracanthum C4R17-cc Date: 28-07-2009 Time: 12:11:21 Particles: 5013 Acq.-Time: 64 s
G1% S% G2M% CV%G1 CV%G2M Mn G2M Mn G1 G2M/G1 ChiSqu.
80.85 15.00 4.16 4.21 3.88 196.31 97.43 2.015 0.72
PK1
PK2
counts
FL4
Region Gate Ungated Count Count/ml %Gated Mean-x CV-x% Mean-y CV-y%
PK1 <None> 3382 3382 - 67.46 98.07 4.84 - -
PK2 <None> 341 341 - 6.80 199.05 6.79 - -
Speed: 0.5
Enable Parameter Gain Log L-L U-L
FL4 340.0 lin 55.0 999.9

Thus, we have analysed by flow cytometry 37 accessions belonging to the species *C. arietinum, C. anatolicum, C. canariense, C. flexuosum, C. macracanthum, C. microphyllum, C. multijugum, C. nuristanicum, C. oxyodon and C. songaricum*, where some of them had been identified differently or tentatively in the past. In these studies, all materials were analysed simultaneously with one or two internal standards (pea and/or *Medicago truncatula*), and with various fluorochromes to assess the relative nuclear DNA content and genome size. For some of the species studied this is the first report on these traits. Fig 4 shows an example of *C. macracanthum* (C4R17) that had been determined to belong to *C. acanthophyllum* by Dr. van der Maesen. Fig 5 gives an overview of all the species/accessions surveyed in this study showing that the C-DNA profile of this '*macracanthum*' plant (red arrow) clearly differed from 'true' *macracanthum* plants (yellow arrow).

Fig. 5. C-DNA values of perennial *Cicer* species. Red arrow indicates a plant that was received as *C. macracanthum* and later identified as *C. acanthophyllum* on the basis of

botanical characteristics. Yellow arrow indicates C-DNA values of true *C. macracanthum* plants.



3.2. Seed increase

Seed collection was originally done by bulking all seeds from the same species. Since a number of plants in our collection were misidentified or deviated, collection started on a single plant basis in 2008. A considerable amount of those seeds were used to grow plants for the *Ascochyta* field trials in 2008 and 2009. In 2009, more than half of the plants in our collection had zero or fewer than 20 seeds stored at the Plant Gene Resources (Agriculture & Agri-Food Canada). If this project will not continue, the 542 perennial plants on the University of Saskatchewan North Seed Farm will be destroyed. Since it is difficult to obtain more seeds from world collections due to the scarcity of the seed supply, the listing of *Cicer* species in the high priority collection list (see Table 2), and the difficulty of growing perennials in many parts of the world, we recommend that another seed collection will be carried out in 2010 at the North Seed Farm. Funding for one summer student will be requested from the Saskatchewan Pulse Growers for this task. All seeds will be permanently stored at the Plant Gene Resources Centre of Canada (AAFC) in Saskatoon.

Table 2. List of high priority *Cicer* taxa as determined by CGIAR/CIAT. Species currently growing at the North Seed Farm are highlighted in red.

- *C. acanthophyllum*
- *C. chorassanicum*
- *C. flexuosum*
- *C. floribundum*
- *C. graecum*
- *C. incisum*
- *C. macracanthum*
- *C. montbretii*
- *C. multijugum*
- *C. nuristanicum*
- *C. oxyodon*
- *C. pungens*
- *C. rechingeri*
- *C. songaricum*
- *C. yamashitae*

3.3. Testing species for *Ascochyta* blight resistance

A systematic design was used to study the reaction to ascochyta blight in perennial chickpea in the field during 2008 and 2009. Seeds from all accessions were seeded in the phytotron and greenhouse starting in February and hardened off prior to transplanting into the field. Seedlings from check cultivars were also transplanted at the same time. Each row of perennials was surrounded on one side by a row of CDC Xena (susceptible check) and on the other side by a row of CDC Frontier (moderately resistant check) (Fig. 6). In 2009, spring in Saskatoon was marked by several late frosts which damaged or killed a number of plants especially from the less cold tolerant species of *C. canariense* and *C. oxyodon*. Transplants were watered every other day until accessions were established. Some accessions were also lost due to drying winds. In order to have a source of inoculum in the field, highly infected plant material was chopped and sprayed into the field. Thereafter, a misting system was used to ensure favorable conditions for disease development. Assessment of foliage disease severity and discolored tissue on stem and leaf were recorded on the whole plant basis using the Horsefall-Barratt scale (Table 3) in late August. The average disease score observed for CDC Xena was 8.45 (8.3 in 2008) and for CDC Frontier was 6.0 (6.3 in 2008), respectively.

Table 3. *Ascochyta rabiei* scores using Horsfall & Barratt (1945) scale

Score	% Diseased	% Healthy
0	0	100
1	0-3	97-100
2	3-6	94-97
3	6-12	88-94
4	12-25	75-88
5	25-50	50-75
6	50-75	25-50
7	75-88	12-25
8	88-94	6-12
9	94-97	3-6
10	97-100	0-3
11	100	0

Fig. 6. Ascochyta blight field trial in 2009 with alternating rows of CDC Xena (susceptible control), perennial accessions and CDC Frontier (control with moderate resistance)

Table 4. SAS Proc mixed analysis/means comparison (LSD) for 2008 and 2009

Year	*Cicer* species	ID	Estimate	LSD
2008	CDC Xena	CX2	9.0	A
2008	CDC Frontier	CF1	6.3	B
2008	*anatolicum*	599110	6.5	ABCDEFGHI
2008	*microphyllum*	593718	4.9	CJ
2008	*microphyllum*	599093	4.6	CDJ
2008	*microphyllum*	599083	4.4	CDEFJ
2008	*microphyllum*	532928	4.4	DEK
2008	*songaricum*	599074	4.2	DEK
2008	*oxyodon*	599084	4.1	DEKL
2008	*microphyllum*	599082	4.0	DEFGHK
2008	*multijugum*	11516	4.0	EFGK
2008	*oxyodon*	561084	3.9	EFGK
2008	*flexuosum*	14524	3.8	EFGHIK
2008	*macracanthum*	599079	3.8	EFGHIK
2008	*multijugum*	599085	3.8	FGHM
2008	*microphyllum*	593719	3.7	FGHILN
2008	*songaricum*	599053	3.7	FGHM
2008	*macracanthum*	599080	3.5	GHIMN
2008	*nuristanicum*	604497	3.4	HIMN
2008	*microphyllum*	599088	3.4	HIMN
2008	*canariense*	557453	3.4	INO
2008	*macracanthum*	599081	3.3	IMN
2008	*anatolicum*	561078	3.0	N
2008	*anatolicum*	599087	2.8	JKLMNOP
2008	*oxyodon*	561103	1.9	P
2008	*anatolicum*	383626	1.9	P

Year	*Cicer* species	ID	Estimate	LSD
2009	CDC Xena	CX2	8.5	A
2009	CDC Frontier	CF1	6.0	B
2009	*microphyllum*	599083	5.3	C
2009	*nuristanicum*	604497	5.2	CD
2009	*multijugum*	599085	5.1	CD
2009	*oxyodon*	561084	4.8	CDE
2009	*songaricum*	599074	4.6	CDEFG
2009	*microphyllum*	599093	4.5	EF
2009	*songaricum*	599053	4.4	EFG
2009	*oxyodon*	561103	4.4	EFG
2009	*oxyodon*	599084	4.3	FG
2009	*microphyllum*	599088	4.2	FG
2009	*microphyllum*	593718	4.2	FG
2009	*multijugum*	11516	4.1	G
2009	*flexuosum*	14524	3.8	GH
2009	*macracanthum*	599080	3.7	DEFGHI
2009	*microphyllum*	599082	3.3	HJ
2009	*microphyllum*	532928	3.3	HJ
2009	*canariense*	557453	3.1	HI
2009	*microphyllum*	593719	2.8	I
2009	*macracanthum*	599079	2.7	IJ
2009	*macracanthum*	599081	2.5	I
2009	*anatolicum*	561078	2.3	IK
2009	*anatolicum*	599110	1.9	K
2009	*anatolicum*	383626	1.8	K

Pearson correlation between 2008 and 2009: r = 0.60819 at *P*= 0.0010

Table 5. SAS Proc mixed analysis/means comparison (LSD) for combined data

Year	*Cicer* species	ID	Estimate	LSD
Combined	CDC Xena	CX2	8.7	A
Combined	CDC Frontier	CF1	6.1	B
Combined	*microphyllum*	599083	4.8	C
Combined	*microphyllum*	599093	4.5	CD
Combined	*microphyllum*	593718	4.5	CDE
Combined	*multijugum*	599085	4.4	CDE
Combined	*songaricum*	599074	4.4	CDEF
Combined	*oxyodon*	561084	4.4	CDEF
Combined	*nuristanicum*	604497	4.3	DEFG
Combined	*anatolicum*	599110	4.2	CDEFGHIJK
Combined	*oxyodon*	599084	4.2	EFGH
Combined	*songaricum*	599053	4.0	FGHI
Combined	*multijugum*	11516	4.0	HI
Combined	*microphyllum*	532928	3.8	HI
Combined	*flexuosum*	14524	3.8	HI
Combined	*microphyllum*	599088	3.8	I
Combined	*microphyllum*	599082	3.7	IJ
Combined	*macracanthum*	599080	3.6	GHIJKL
Combined	*macracanthum*	599079	3.3	JKLM
Combined	*canariense*	557453	3.2	JKLM
Combined	*microphyllum*	593719	3.2	KLM
Combined	*oxyodon*	561103	3.2	KLM
Combined	*macracanthum*	599081	2.9	LMN
Combined	*anatolicum*	561078	2.7	N
Combined	*anatolicum*	599087	2.2	MNO
Combined	*anatolicum*	383626	1.8	O

Data were statistically analyzed using a SAS Proc mixed analysis and a LSD means comparison carried out for the 2008, 2009 (Table 4) and combined data (Table 5). The analysis showed that *Ascochyta* rating for each accession was closely related in both years (Pearson correlation between 2008 and 2009: r = 0.60819 at *P*= 0.0010). Combined LSD values demonstrate that both controls were different from all perennials i.e. all wild accessions had significantly better ascochyta blight resistance ratings than either control. *Cicer anatolicum* PI 383626 had the best blight resistance rating of 1.8 under heavy disease pressure and no spraying (Fig. 6 red arrow). In contrast, our current best cultivar, CDC Frontier, has a resistance rating of only 6.1 indicating that the perennial species have considerable scope for improving blight resistance in chickpea.

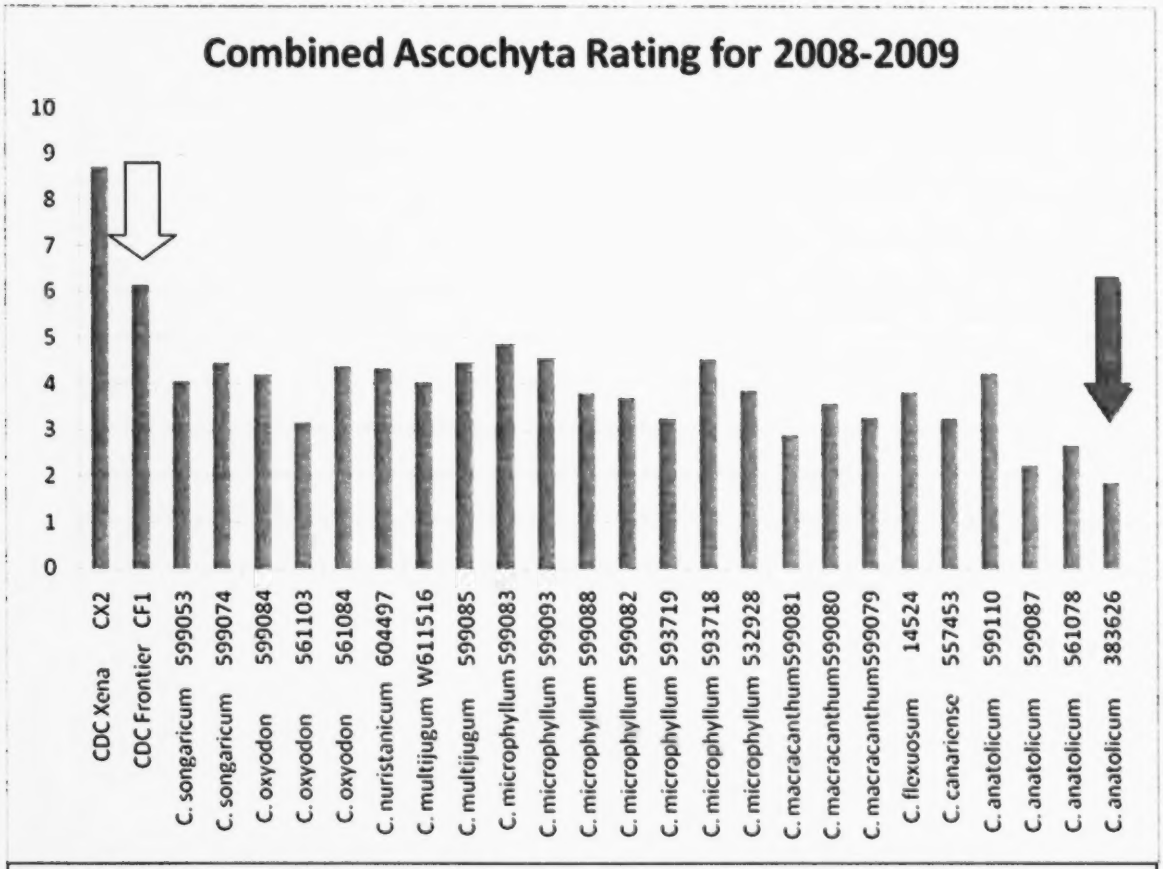


Fig. 6. Overview of *Ascochyta rabiei* resistance rating for the different perennial accessions compared to the chickpea control cultivars CDC Xena (susceptible control) and CDC Frontier (moderately resistant control; yellow arrow). All accessions were significantly different from the controls.

A new two-year project led by Dr. S. Banniza on 'Infection studies of *Ascochyta rabiei* on wild chickpea germplasm' is currently underway to determine if the blight resistance mechanism in the wild species is different from the one in the cultivated species. If this study shows that the mechanism of disease resistance is durable and/or different, then efforts to continue incorporating this resistance into the cultivated chickpea are warranted. This two year project is funded by the Saskatchewan Pulse Growers.

3.4. Improvements to the hybrid rescue protocol

In order to obtain hybrid plants, two major issues were targeted this year; first, improving the hybrid pod rescue protocol and second, finding ways to reduce the losses of cultured pods as a result of fungal and/or bacterial contamination. Hybrids from crosses with *C. anatolicum* and *C. oxyodon* were rescued 5 DAP, whereas embryos from crosses involving *C. macracanthum* and *C. microphyllum* could be left for up to10 days.

3.4.1. Medium improvements

Raghaven (2003) stated that 'early stage embryos require complex exogenously supplied metabolites to maximize their chances for survival and growth, whereas late stage embryos need only a relative simple medium consisting of mineral salts and a carbon energy source such as sucrose'.

Table 6. Composition of base media

Macro in mg/L	**PC[1]**	**NMM[2]**	**TM[3]**
NH_4NO_3	1,000	1,000	
KNO_3	2,100	2,100	
KCl			746
$CaCl_2 \cdot 2H_2O$	600	600	
$CaSO_4 \cdot 2H_2O$			515
$MgSO_4 \cdot 7H_2O$	435	435	370
KH_2PO_4	325	325	170
$NaH_2PO_4 \cdot H_2O$	85	85	
Micro mg/l			
KI	1.00	1.00	0.83
H_3BO_3	5.00	5.00	6.20
$MnSO_4 \cdot H_2O$	15.00	15.00	16.91
$ZnSO_4 \cdot 7\ H_2O$	5.00	5.00	8.60
$Na_2MoO_4 \cdot 2H_2O$	0.40	0.40	0.25
$CuSO_4 \cdot 5H_2O$	0.10	0.10	0.025
$CoCl_2 \cdot 6H_2O$	0.10	0.10	0.025
NaFeEDTA	25.00	25.00	27.85
Vitamins			
Myo-Inositol	250.00	250.00	100.00
Pyridoxine HCl	0.50	0.50	0.40
Nicotinic acid	1.00	1.00	0.50
Thiamine HCl	2.00	2.00	0.40
Ca pantothenate	1.00	1.00	
Vit. D_3Cholecalciferol	0.10	0.10	
Cefotaxime	150.00	150.00	150.00
Coconut water (ml)	70.00	70.00	
Glycine			40.00
Amino acids			
Casein hydrol.	300.00	300.00	
Glutamine g/l			9.13
Sucrose	90.00	50.00	50.00
pH	5.80	5.80	6.00

[1]Phillips and Collins 1979
[2] Mallikarjuna 1999
[3]Thompson et al 1977 and Singh & Kaushal 2000

To achieve the transition from the globular to the heart-shaped stage is a major challenge at this point. Last year, a medium (PC) based on Phillips and Collins (1979) was used for pod culture but regeneration rates were low and hybrids could not be recovered. A medium developed by Mallikarjuna (2001) from ICRISAT for rescue of *C. arietinum* x *C. bijugum* crosses was used as control. Hence, efforts continued to improve the rescue medium.

Experiment 1:

Objective: Optimizing plant growth conditions by determining which factors have an effect on pod set. If we can optimize parent plant growth conditions so that fewer flowers abort and more ovules continue to grow, than we increase our chances of regenerating hybrids.
Design: Chickpea flowers were routinely tagged on Tuesday, Wednesday and Thursday since the pods could then be rescued on the following Monday, Tuesday and Wednesday i.e. 5 days after fertilization. The number of pods was then counted. Table 7 shows an example of pod development for three parent plants.
Results:

- Pod set in chickpea is generally low even under good growth conditions i.e. only 24% of all flowers tagged produced pods
- Pod set is influenced by the individual plant. Some plants (Plant B) generally had poorer pod set rates (10%) compared to other plants (28% and 31%) even though they were grown on the same bench and treated in the same way. The only difference was that the soil of Plant B was wetter than the other two pots.
- Depending on the day, more flowers set pods (22.4) than on others (21.4) even though all three days were sunny and the temperature setting in the greenhouse remained the same.
- Parent plants need to be in optimum condition. Stress caused by over- or under-watering negatively affects pod set rates and regeneration.

Table 7. Influence of parent plant on selfed pod development

Date	**Plant A**		**Plant B**		**Plant C**		**Total No of**		**Percentage**
2009	Flowers tagged	No of Pods	Flowers tagged	No of Pods	Flowers tagged	No of Pods	Flowers tagged	Pods	Flowers/ Pods
20.4	12	3	16	1	15	6	43	10	23
21.4	16	2	9	1	12	2	37	5	14
22.4	11	6	5	1	12	4	28	11	39
Total	**39**	**11**	**30**	**3**	**39**	**12**	**108**	**26**	**24**

Experiment 2:

Objective: Determine medium effect on pod number and ovule size

Design: Flowers were tagged at anthesis, resulting pods removed after 5 days, sterilized and randomly transferred to various media (Table 8). After two weeks, ovules were dissected out and measured.
Results:

- Percentage of pods with ovules varied widely (0 to 59%) indicating that parent plants have a confounding effect on medium screening
- Medium NMM-90 and TM-7+ are not suitable since ovules were callusing or otherwise abnormal
- Finding a live thrip in a pod indicates that thrips are carried from the greenhouse into culture and can cause widespread contamination
- Ovule size at initial culture was 1 - 2 mm indicating that many ovules did not continue to develop in culture
- Since ovule size and embryo size is correlated, the larger the ovule size the further the embryo has developed
- Base medium TM-O was found to result in larger ovules without abnormalities

Table 8. Influence of parent plant and medium on ovule size

Medium	% Pods with Ovules	Ovule Size (mm)						Comment
		1	2	3	4	5	6	
NMM-90	19	+	+	+	+	-	-	abnormal
NMM-30	0	-	-	-	-	-	-	
TM-7+	50	+	-	-	-	-	-	abnormal
TMO+Spd+GA_3	39	+	+	-	-	+	+	
TM4+Spd+GA_3	59	+	+	-	+	+	-	1 thrip alive
TM-O	14	-	+	-	+	+	-	
TM-4	14	-	-	+	+	-	-	
TMO + Glu	20	-	-	+	+	+	+	
TM4 + Glu	17	+	+	-	+	+	+	

Experiment 3:

Objective: Develop pod culture medium using TM-O as base and NMM-90 as control and determine if culture vessel (short or long tubes versus Magenta boxes) has an influence on ovule growth
Design: Flowers were tagged at anthesis, resulting pods removed after 5 days, sterilized and randomly transferred to various media (Table 9) and containers. Each tube (long or short) contained 4 pods whereas each Magenta box contained 12 pods. After four weeks, ovules were dissected out and measured followed by dissection of embryos out of the ovule. Note that embryos could not be dissected out of ovules 4 mm or smaller (5 mm was borderline) using a dissecting microscope.
Results: Data presented in Table 10 confirmed that NMM-90 (control) was not a suitable medium for pod culture regardless of the culture vessel. There was no

consistent difference between the TMO medium and its variations. Magenta boxes were found not suitable for hybrid rescue due to frequent contamination and thus major losses. The length of the culture tubes also had no consistent influence. On average, 45% of pods with ovules produce embryos on TMO or TMO variations (Fig 7 and 8).

Table 9. Media used for pod culture

Macro in mg/L	**TM0**	**TM0-B1**	**TM0-B2**	**TM0-B3**	**TM0-B4**
KCl	746	746	746	746	746
$CaSO_4 \cdot 2H_2O$	515	515	515	515	515
$MgSO_4 \cdot 7H_2O$	370	370	370	370	370
KH_2PO_4	170	170	170	170	170
Micro					
NaFeEDTA	27.85	27.85	27.85	27.85	27.85
KI	0.83	0.83	0.83	0.83	0.83
H_3BO_3	6.20	6.20	6.20	6.20	6.20
$MnSO_4 \cdot H_2O$	16.91	16.91	16.91	16.91	16.91
$ZnSO_4 \cdot 7\ H_2O$	8.60	8.60	8.60	8.60	8.60
$Na_2MoO_4 \cdot 2H_2O$	0.25	0.25	0.25	0.25	0.25
$CuSO_4 \cdot 5H_2O$	0.025	0.025	0.025	0.025	0.025
$CoCl_2 \cdot 6H_2O$	0.025	0.025	0.025	0.025	0.025
Vitamins					
Myo-Inositol	100	100	100	100	100
Pyridoxine	0.50	0.50	0.50	0.50	0.50
Nicotinic acid	0.50	0.50	0.50	0.50	0.50
Thiamine HCl	0.40	0.40	0.40	0.40	0.40
Amino Acids					
Glutamine g/l	9.13	9.13	9.13	9.13	9.13
Glycine	40	40	40	40	40
Casein hydroly		300	300	300	300
Proline mg		600	600	600	600
Glutathione		90	90	90	90
Larcoll mg/l		50	50	50	50
Cefotaxime	150	150	150	150	150
Carbohydrates					
Sucrose g/l	**50**	**50**	**50**	**50**	**50**
Putrescine		**160**	**250**	**500**	**100**
pH	6.00	6.00	6.00	6.00	6.00

Table 10. Testing effect of various media and culture vessels on pod culture, ovule and embryo development

Medium	# of Pods	Pods with ovules	Ovules 2-3mm	Ovules 4-5mm	Ovules 6-7mm	Ovules > 7mm	No. of Embryos	% Embryos per pod
Long tubes								
NMM-90	64	26	32	4	0	0	4	15
TMO-B1	104	41	39	4	8	0	7	17
TMO-B2	132	47	30	15	15	1	20	43
TMO-B3	92	34	31	5	12	1	11	32
TMO-B4	120	22	10	3	13	0	13	59
TMO	113	18	11	3	6	2	8	44
Magenta								
NMM-90	84	13	2	1	2	0	3	23
TMO-B1	72	6	2	2	0	0	1	17
TMO-B2	72	6	2	4	1	0	2	33
TMO-B3	84	7	2	3	1	1	4	57
TMO-B4	74	10	n.d.	4	4	0	8	80
TMO	84	3	0	0	3	0	3	100
Short tubes								
NMM-90	152	50	70	2	0	0	0	0
TMO-B1	76	15	23	3	5	1	6	40
TMO-B2	83	22	25	5	8	0	10	45
TMO-B3	104	31	28	6	5	0	8	26
TMO-B4	112	27	8	6	12	2	10	37
TMO	192	58	31	24	16	3	21	36

3.4.2. Reducing contamination rates

Contamination of cultures was a major problem in 2008 and resulted in losses of many hybrid crosses. In order to improve our chances of obtaining hybrids, we needed to find a more effective method for sterilizing pods.

Objective: Reducing contamination rates in pod culture caused by bacteria and fungi

Design: Testing various concentrations of bleach and type of sterilization methods (chemical: bleach, dichloroisocyanuric acid (DCICA), calcium hypochlorite (TNT); light: (ultraviolet) on pods from selfed chickpea.

Fig 7. Pod from selfed chickpea flower after culture on TM-O for 4 weeks

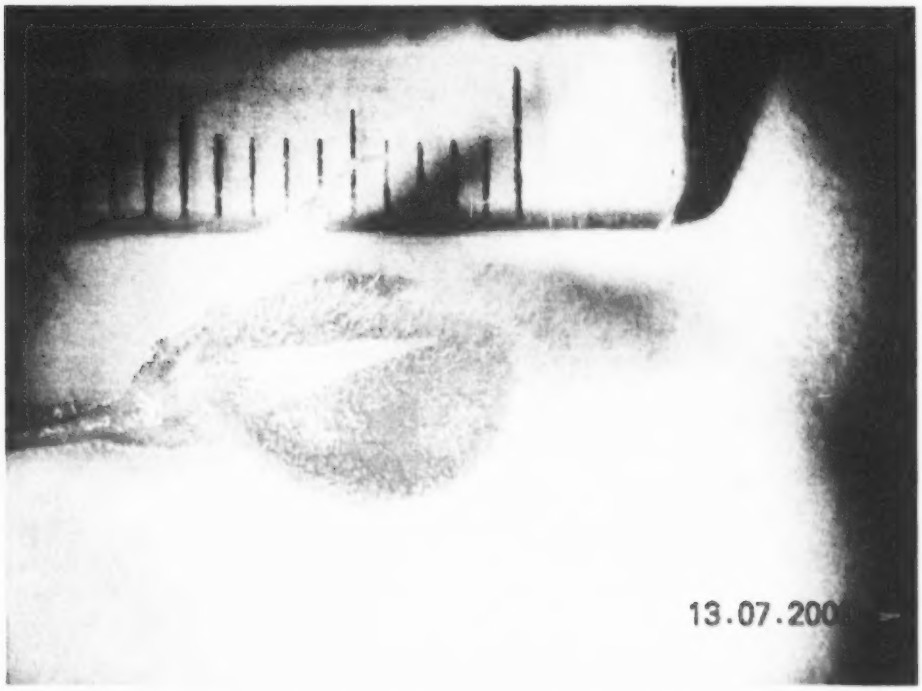
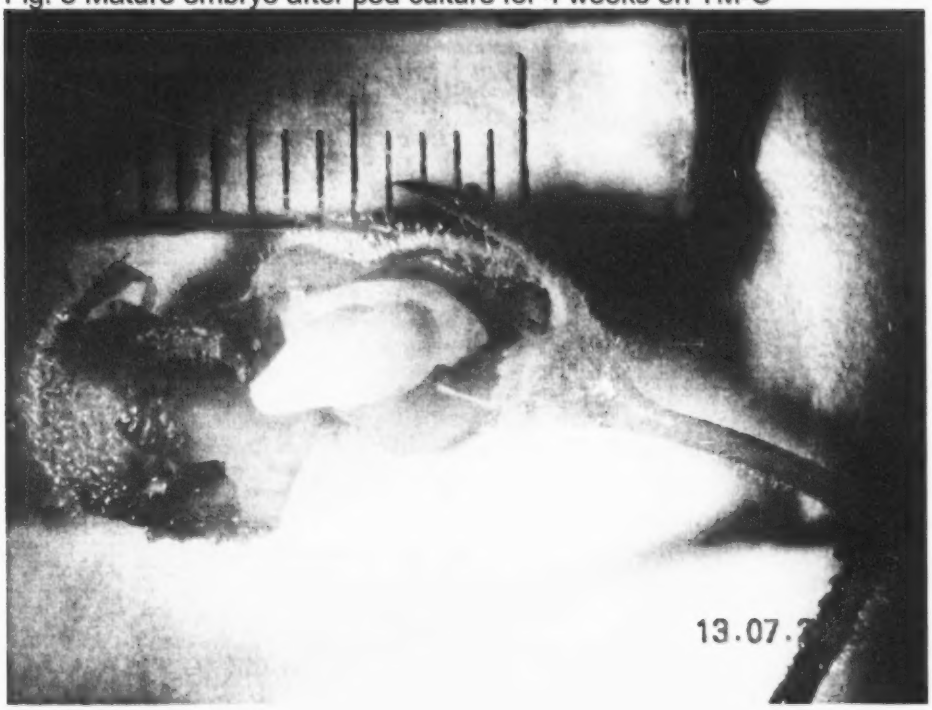


Fig. 8 Mature embryo after pod culture for 4 weeks on TM-O



Results: No procedure resulted in 100% clean cultures. Using a buffered bleach solution resulted in lower contamination rates and less tissue damage. Combining bleach and UV light was slightly better but the method was more time consuming. Unfortunately, this technique developed with pods from greenhouse grown material was not sufficient for pods from the polyhouse. The latter material had much higher contamination rates.

3.5. Crossing with wild species

To date no confirmed hybrids have been reported between the cultivated and perennial chickpea. Babb and Muehlbauer (2005) reported that in crosses of Myles x *C. anatolicum* fertilization occurs, but the resulting zygote and endosperm nucleus are unable to divide to generate an embryo and endosperm. In contrast to our experiments, these crosses were carried out without application of hormones. Our microscopic examinations (see 2008 report) showed that hybrid embryos developed from crosses between the cultivated and perennial chickpea species to various stages. Crossing chickpea with *C. macracanthum* resulted in globular to heart-shaped embryos; crossing with *C. oxyodon* produced globular embryos; crossing with *C. microphyllum* resulted in globular to mature embryo; but crossing with *C. songaricum* only led to zygote development.

Objective: This year, we had two major objectives. First, focus on crossing with *C. anatolicum* PI383826 and *C. oxyodon* 561103 because of their high ascochyta blight resistance. Second, focus on crosses with *C. macracanthum* and *C. microphyllum* in order to produce hybrids that might be useful for bridge crossing.

Experimental Design: Crossing was carried out in the polyhouse as in previous years using ICCV10 as female parent. Pollen originated from individual plants because of possible misidentification of species and each source was recorded. Hormone application was carried out as in previous years. Crosses involving C. *anatolicum* and *C. oxyodon* were rescued 5-6 days after pollination, whereas crosses with C. *macracanthum* and *C. microphyllum* were rescued 9 - 10 days after pollination. Tagged pods were sterilized using UV light and bleach before transferring them to culture tubes with filter-paper bridges and liquid TM-O medium.

Results: A total of 3,429 crosses were made using *C. anatolicum* PI383626, *C. oxyodon* PI561103, *C. macracanthum* PI 599079 and *C. microphyllum* PI 593719. 2197 pods were transferred to TM-O medium i.e. 64% of all crosses resulted in pod development. However, subsequent losses due to contamination continued to be a major problem. Even though UV light combined with bleach sterilization considerably reduced contamination rates from greenhouse grown material, it was not effective for crosses grown in the polyhouse. More than 60% of crosses were lost as a result of fungal and mold contamination. Why the contamination rate in polyhouse grown material was so much more severe

remains unclear. It is possible that the soil contained higher amounts of spores compared to Sunshine No. 4 used in the greenhouse and that the rain which leaked through the roof splashed spores from the soil onto the plants. We clearly need to improve the survival rates of pods in culture in order to improve our chances of obtaining hybrid crosses.

The remaining pods were kept on TM-O medium for four weeks. Then, ovules were dissected out and measured. Results showed that ovules were still only 1-2 mm in size indicating that embryo development had ceased. This was in contrast to the previous year where a small number of ovules had enlarged. No hybrid plants could be regenerated.

3.6. Conclusions

Cultivated chickpea is hampered by low levels of genetic diversity within the species. In contrast, wild related *Cicer* species possess a wealth of genes not only for *Ascochyta* blight resistance but also for many other agronomically important traits such as *Fusarium* wilt resistance, cold and drought tolerance.

Results from our two-year field screening clearly showed that all perennial species have *Ascochyta* blight resistance superior to what is available in our cultivars even under high disease pressure and no fungicide application. *C. anatolicum* PI 383626 received the best resistance rating of 1.8.

Yet, incorporating this resistance into the cultivated species has proven difficult similar to reports from USDA and ICRISAT researchers. It is clear from our microscopic analyses that hybrid embryo development to the globular or heart-shape stage had taken place but continued growth could not be facilitated. In order to move past this bottleneck we need to improve our rescue protocol, reduce contamination rates as well as optimize our rescue medium. We had planned to use the hormone analysis of developing selfed ovules as a basis for improving our medium. However, this experiment could not be carried out in the spring of 2009 due to labour shortages and infrastructure problems.

A new project on 'Infection studies of *Ascochyta rabiei* on wild chickpea germplasm' funded by the Saskatchewan Pulse Growers is currently underway. This study will determine whether the resistance mechanism in perennial species is very durable and thus more desirable (i.e. of a non-host-type), based on a gene-for-gene interaction (i.e. relatively less durable) as in our current cultivars, or has a completely different mechanism altogether.

If the outcome of the infection study shows that the resistance found in perennial chickpea is very durable and the hormone analysis of young ovules leads to an improved rescue protocol than efforts should continue to transfer blight resistance from wild perennial to the cultivated species.

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E. PERSONNEL

Drs. M. Lulsdorf and A. Diederichsen were the research scientists working on this project. *Ascochyta* ratings were performed by Ms. P. Hashemi. Regarding summer students, Ms Tamasyn Ament worked from May 1st to August 31st; Ms Tana Karchewski worked part-time during the summer and Mr Shea Kadyschuk worked from June to August 31st.

F. EQUIPMENT

No equipment was purchased for this project.

G. PROJECT DEVELOPED MATERIALS

1. This project was presented as a poster 'Two wild relatives of the cultivated chickpea as potential sources of resistance to *Ascochyta* blight' at Pulse Days 2009.
2. A poster on 'High levels of *Ascochyta* blight resistance found in wild species' is planned for Pulse Days 2010.
3. A conference paper on 'Challenges in preserving, communicating and utilizing the diversity of wild perennial species of the genus *Cicer* L.' was presented at St. Petersburg, Russia, November 16-19, 2009.
4. A poster for the conference on 'Legumes for Global Health' in Turkey in April 2010 has been submitted with the title 'DNA content and genome size of some perennial *Cicer* species'.
5. A short publication in Cytometry A on '*Cicer* taxonomy: a flow cytometry approach' is in preparation
6. A publication of the results from this project including the 2-year ascochyta study and all the microscopy work is also planned for 'Plant Genetic Resources'.

H. PROJECT PHOTOS

See text

I. ACKNOWLEDGEMENTS

The Saskatchewan Agricultural Development Fund and the Saskatchewan Pulse Growers will be acknowledged in all posters or presentations where this research project is presented. We also gratefully acknowledge receiving accessions from Dr. Fred Muehlbauer (USDA-USR Pullman), Dr. Nalini Mallikarjuna (ICRISAT India), IPK and VIR.

J. EXPENSE STATEMENT

Will be provided separately.